2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

ACC Reference Number: OLF-63.0-HPV3-HLS

Data requirement:	EC Directive 92/69, Part C3 OECD Procedure No. 201 US EPA TSCA 797.1050 & 797.1060
Huntingdon Life Sciences Limited Project Identity:	CSS/003
Study completed on:	22 April 2004

Sponsor

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice Standards and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No. 3106).

EC Commission Directive, 1999/11/EC of 8 March 1999 (Official Journal No. L 77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

United States Environmental Protection Agency (TSCA) Title 40 Code of Federal Regulations, Part 792, Federal Register, 17 August 1989.

A retention sample of the test substance was not taken as it was not indefinitely stable and posed a safety hazard.

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Carole A. Jenkins, B.Sc.(Hons.), C.Biol., M.I.Biol., M.I.F.M.
Study Director,
Huntingdon Life Sciences Ltd.

22 April 2004
Date

This final report was accepted on the behalf of the Sponsor

.....

Elizabeth J Moran
Sponsor's Representative

1 April 2004
Date

QUALITY ASSURANCE STATEMENT

The following inspections and audits have been carried out in relation to this study.

Study Phase	Date of Inspection	Date of Reporting
Protocol Audit	17 September 2001	17 September 2001
Study Based Inspections		
Experimental set-up	18 February 2002	18 February 2002
Cell counting	22 August 2002	22 August 2002
Process Based Inspections		
Sampling of test media	8 November 2002	8 November 2002
Sample dilution, preparation & GLC analysis	12 November 2002	13 November 2002
Report Audit	17 March 2003	17 March 2003

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Study Based Inspections: Inspections and audits of phases of this study were conducted and reported to the Study Director and Company Management as indicated above.

Process Based Inspections: At or about the time this study was in progress inspections of routine and repetitive procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

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21 April 2004
.....
Date

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SUMMARY

The effect of 2-methyl-2-butene on the growth of the unicellular green alga *Pseudokirchneriella subcapitata* was assessed under non-axenic (non-sterile) conditions.

The study was conducted in accordance with EC Methods for Determination of Ecotoxicity, Annex to Directive 92/69/EEC (O.J. No. L383A, 1992) Part C, Method 3 - Algal Inhibition Test and the OECD Guideline for Testing of Chemicals No. 201 - Alga, Growth Inhibition Test (7 June 1984) with the following exceptions so that the requirements of the US EPA Code of Federal Regulations, Part 797 – Environmental Effects Testing Guidelines under the Toxic Substances Control Act (1987) could be met: the duration of the test was 96 hours, the test temperature was 24 ± 2°C and the light intensity of the test area was approximately 4000 lux. To accommodate the testing of a volatile substance, the study design deviated from each of these test guidelines by altering the composition and pH of the culture medium; these amendments were necessary to compensate for the lack of headspace for gaseous exchange and to minimise the pH increase during the test.

The range finding and definitive tests were conducted in completely filled (no headspace) and sealed vessels because of the volatility of 2-methyl-2-butene. The test media were prepared, either directly or by dilution, from an aqueous preparation of the test substance, which was stirred for approximately 23 hours in the dark. After being allowed to stand for approximately 1.5 hours to obtain an equilibrium concentration of 2-methyl-2-butene, a portion of the medium was removed from the middle of the vessel and after dilution and inoculation with algal cells, it was used to fill the test vessels. The cultures were incubated in an orbital incubator under continuous illumination at temperatures ranging from 22.3 to 23.4°C for 96 hours.

Replicate algal cultures, with an initial cell density of 1 x 10^4/ml, were exposed to algal nutrient medium (the control) and to 2-methyl-2-butene at nominal concentrations of 3.20, 7.04, 15.5, 34.1 and 75 mg/l. The exposure levels were monitored using a GLC (gas liquid chromatographic) method of analysis. Although lower than intended, the measured concentrations of 2-methyl-2-butene in the test cultures at the start (between 19 and 27% of their nominal values) were adequately maintained during the test, with measured levels of between 22 and 29% of nominal after 96 hours. Based on an arithmetic mean, the overall mean measured levels of 2-methyl-2-butene were 0.689, 1.53, 3.61, 7.22 and 21.1 mg/l.

Cell densities (the number of cells per ml) were counted daily to monitor growth. The test results are expressed in terms of the area under the growth curve (the increase in cell density during the test period) and growth rate (the increase in cell density per unit time) using the mean measured concentrations. The following values were derived from the data:

Area under the growth curve

E_bC_{50} (72 h) : 10.5 mg/l (95% confidence limits, 9.55 & 11.7 mg/l)

E_bC_{50} (96 h) : 10.1 mg/l (95% confidence limits, 9.21 & 11.1 mg/l)

No observed effect concentration (NOEC) : 3.61 mg/l

Average specific growth rate

E_rC_{50} (0 - 72h) : 12.0 mg/l (95% confidence limits, 7.22 & 21.1 mg/l)

E_rC_{50} (0 - 96 h) : 13.2 mg/l (95% confidence limits, 12.2 & 14.3 mg/l)

No observed effect concentration (NOEC) : 7.22 mg/l

Where:

E_bC_{50} ("x"): median effect concentration for inhibition of growth based on areas under the growth curves after "x" hours.

E_rC_{50} ("x" – "y"): median effect concentration for inhibition of growth based on a comparison of growth rates from "x" to "y" hours.

INTRODUCTION

This study was designed to assess the effect of 2-methyl-2-butene on the growth of the unicellular green alga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*).

The study was conducted in accordance with EC Methods for Determination of Ecotoxicity, Annex to Directive 92/69/EEC (O.J. No. L383A, 1992) Part C, Method 3 - Algal Inhibition Test and the OECD Guideline for Testing of Chemicals No. 201 - Alga, Growth Inhibition Test (7 June 1984) with the following exceptions so that the requirements of the US EPA Code of Federal Regulations, Part 797 – Environmental Effects Testing Guidelines under the Toxic Substances Control Act (1987) could be met: the duration of the test was 96 hours, the test temperature was 24 ± 2°C and the light intensity of the test area was approximately 4000 lux. To accommodate the testing of a volatile substance, the study design deviated from each of these test guidelines by altering the composition and pH of the culture medium; these amendments were necessary to compensate for the lack of headspace for gaseous exchange and to minimise the pH increase during the test.

The protocol was approved by Huntingdon Life Sciences Management on 17 July 2001, by the Sponsor on 21 August 2001, and by the Study Director on 14 September 2001.

The study was conducted at Huntingdon Life Sciences Ltd., Eye Research Centre, Eye, Suffolk, IP23 7PX, England.

The experimental start and termination dates for the study were 9 January 2002 and 19 November 2002, respectively.

Information on the boiling point of 2-methyl-2-butene (35 to 38°C) indicated that it was volatile.

The results of the most recent laboratory reference test (30 September 2002) using potassium dichromate indicated that its 72-hour E_bC_{50} to *Pseudokirchneriella subcapitata* was 0.84 mg/l; this was within the range typically obtained in this laboratory (0.3 to 1 mg/l).

Samples of test media taken during this study were analysed with samples taken from another study performed on this test substance; namely Huntingdon Life Sciences Study Number CSS/004 (Acute toxicity to *Daphnia magna*). Consequently, data relating to the chemical calibration standard solutions were shared between the two studies.

TEST SUBSTANCE

Identity:	2-methyl-2-butene
Alternative identity:	2-methyl, 2-butene
CAS number:	513-35-9
Lot number:	A0153320
Stability:	The test substance was analysed in an investigation conducted as part of HLS Study Number CSS/007 (see Appendix 3 for Certificate of Analysis), which showed that it was stable for the duration of testing in this study.
Purity:	> 98% (Appendix 3)
Appearance:	Clear, colourless liquid
Storage conditions:	In a cool, dry, well-ventilated area
Source:	Fisher Scientific UK Bishop Meadow Road Loughborough Leicester LE11 5RG UK
Date received:	16 May 2001

EXPERIMENTAL PROCEDURE

TEST SPECIES

Name

Pseudokirchneriella subcapitata Strain No. CCAP 278/4. Unicellular green algae such as *Pseudokirchneriella subcapitata* are used as model systems in tests to determine whether a chemical affects the primary productivity of plants in the freshwater environment. Since many unicellular green algae have short generation times, this relatively short test can be used to assess the effects of a substance over many generations.

Source

Axenic (sterile), uni-cellular, liquid cultures of algae were obtained from the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, Cumbria, UK and arrived on 25 September 2002. Formal identification of the species was undertaken by the supplier although on receipt at Huntingdon Life Sciences, the culture was examined under the microscope to confirm that it was uni-cellular and not contaminated.

Pre-culture

The liquid cultures were stored in an illuminated refrigerator. Sterile algal nutrient medium (Appendix 1) was inoculated with cells aseptically removed from the liquid culture; these primary liquid cultures (100 ml) were incubated for approximately three days in an orbital incubator under continuous illumination at nominal temperatures in the range 22 to 26°C. An aliquot from one of the primary cultures, characterised by a cell density of 0.84×10^6 cells/ml, was aseptically transferred to fresh sterile algal nutrient medium to prepare a secondary liquid culture; this culture was incubated, as stated above, for a further three days to provide an inoculum in the log phase of growth, characterised by a cell density of 1.72×10^6 cells/ml. An aliquot of one of the secondary culture was used in the definitive test to give an initial cell density of 1×10^4 cells/ml in the control and test vessels.

CULTURE MEDIUM

The culture medium was sterile algal nutrient medium, as recommended in Official Journal No. L383A Part C.3 and OECD Procedure 201 (see Appendix 1). Because the test was conducted in completely filled and sealed vessels, with no headspace for gaseous exchange, the concentration of sodium bicarbonate in the culture medium was increased to 300 mg/l and to compensate for this, the pH of the culture medium was adjusted to approximately 7 before use.

TEST SUBSTANCE PREPARATION

Method of preparation (definitive test)

The method of formulation used was based on the results of a formulation trial and the range finding test.

The test media were prepared either directly or by dilution from an aqueous preparation with a nominal concentration of 75 mg/l. An amber glass bottle (total capacity, *ca.* 2.68 l) was completely filled with culture medium and sealed using a screw cap fitted with a silicone septum. The contents of the preparation vessel were stirred at a rate capable of creating a vortex of between 0 and 5% of the static depth of the medium in the vessel before it was sealed. The test substance (nominally 305µl) was injected through the silicone septum into the stirring dilution medium. The preparation vessel was covered with a black bag and left to stir for approximately 23 hours. On cessation of stirring, the preparation vessel was left to stand for approximately 1.5 hours and after samples were taken for chemical analysis, aliquots of the medium were removed from the preparation vessel and placed into volumetric flasks. After the addition of the algal inoculum (5.2 ml), the medium was used either to fill the test vessels at the highest test concentration or was diluted with culture medium to provide the test media at the lower concentrations.

Stability of test concentrations

The test concentrations of 2-methyl-2-butene were measured using a GLC method of chemical analysis (see Appendix 2). For the definitive test, two sample bottles (glass reaction vials, nominal capacity 10 ml) were completely filled with medium taken from the aqueous preparation at 75 mg/l before it was used to prepare the test media at the lower concentrations. After the vessels at each concentration were filled with freshly-prepared control or test medium, two sample bottles were completely filled using the medium remaining in each of the preparation vessels. After the samples for cell counting at 96 hours were removed, two of the three vessels from each exposure group were retained for analysis. One sample (*ca.* 10 ml) was removed from each vessel and was used to completely fill a glass vial. On each occasion, all of the samples in bottles were placed into a refrigerator until analysis (after storage for either 23 or 24 days). On completion of the study, all of the samples were analysed.

Additional samples were also taken from test vessels containing 2-methyl-2-butene at 3.20 and 75 mg/l but with no algal cells, in order to obtain information on the extent of adsorption/absorption of the test substance by the algal cells.

EXPOSURE CONDITIONS

Experimental design

The study was conducted in completely-filled, sealed vessels under static exposure conditions and comprised a formulation trial, a range finding test and two main tests, the first of which was considered invalid because measurable levels of the test substance were found in control samples. The second main test (hereafter referred to as the definitive test) employed five test concentrations plus one control (dilution medium).

The formulation trial was performed to identify a suitable technique for the addition of the test substance to the dilution medium and to determine the time taken for the test substance to reach equilibrium in the dilution medium. Chemical analysis of samples of the media taken approximately 3, 24 and 48 hours after preparation, indicated that approximately 24 hours of mixing was adequate to provide a stable dilution.

Using the procedures identified in the formulation trial, the range finding test was conducted using triplicate cultures; the cells were exposed to the test substance at nominal concentrations of 0.5, 5 and 50 mg/l. After 96 hours, cell growth in cultures at 50 mg/l had been inhibited by approximately 55% compared to control cultures; at 0.5 and 5 mg/l, cell growth was similar to that found in control cultures. Measured concentrations of 2-methyl-2-butene in samples taken from the test cultures ranged between 28 and 41% of their nominal values. To verify that the design of the test vessels was suitable for use with the testing of a volatile substance, glass bottles were also completely filled with control or test medium and sealed at the start of the test and incubated with the test flasks; after 96 hours, measured levels of 2-methyl-2-butene in samples taken from the glass bottles (between 27 and 43% of their nominal values) were similar to those found in the test flasks confirming that the test flasks were suitable for use.

Based on these findings, the definitive test employed nominal concentrations of 3.20, 7.04, 15.5, 34.1 and 75 mg/l plus an algal nutrient medium control group. Twelve flasks were established for each control and test group; in addition, one extra flask each was prepared at 3.20 and 75 mg/l and filled with test medium but no algal cells. All of the control and test flasks were incubated; the medium remaining in each of the preparation vessels was used for water quality measurements and chemical analysis at the start.

Before the start of the test, the required number of empty test vessels (*ca.* 65 ml conical flasks), were loosely stoppered with non-absorbent cotton wool, covered with aluminium foil which was secured by autoclave tape and sterilised by autoclaving (121°C for 15 minutes). Each vessel was completely filled with control or test medium and was sealed with a glass stopper, leaving no headspace, which was then covered with laboratory film (Parafilm®). The vessels were labelled with the HLS study number, a unique vessel number and the nominal exposure concentration. The initial cell density in each flask was approximately 1 x 10^4 cells/ml.

The control cultures were prepared by adding aliquots (*ca.* 65 ml) of inoculated culture medium to each flask.

Environmental conditions

Conical flasks (*ca.* 65 ml) containing control or test cultures were placed in an illuminated orbital incubator according to a random number sequence. The cultures were incubated, without renewal of medium for 96 hours under continuous illumination of 3430 and 4360 lux, which was provided by 6 x 30 W "cool white" 1 metre fluorescent tubes. The temperature was maintained at nominal 24 ± 2°C.

Temperature and pH of control and test flasks at the start and end of the test were recorded. Gaseous exchange and suspension of the algal cells were ensured by the action of the orbital shaker, oscillating at a nominal 150 cycles per minute. The minimum, maximum and ambient temperature and light intensity in the test area were determined each day.

MEASUREMENT OF GROWTH

The number of cells per ml (cell densities) were measured using a haemacytometer (Improved Neubauer) at approximately 24, 48, 72 and 96 hours. On each occasion, samples were taken from three control flasks and three test flasks at each concentration; the flasks were discarded after the samples had been removed except at 96 hours when samples were taken for chemical analysis and water quality measurements. The estimate of cell numbers in each sample was based on the mean of four or eight consecutive counts depending on the cell density of the cultures. The presence of any abnormal cells was also noted during counting.

EVALUATION OF DATA

The area under each growth curve (cell density *versus* time) is taken to be an index of growth and is calculated using the equation:

$$A = \frac{N_1 - N_o}{2} \times t_1 + \frac{N_1 + N_2 - 2N_o}{2} \times [t_2 - t_1] + \ldots + \frac{N_{n-1} + N_n - 2N_o}{2} \times [t_n - t_{n-1}]$$

where A = area
N_o = nominal cell densities at t_o
N_1 = measured cell densities at t_1
N_n = measured cell densities at t_n
t_1 = time of first measurement (hours from start)
t_n = time of n_{th} measurement (hours from start)
n = number of measurements taken after the beginning of the test

Percentage inhibition of growth at each test concentration (I_A) was calculated by comparing the area under the test curve (A_t) with that under the control curve (A_c) as appropriate using the equation:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

The E_bC_{50} ("x" h) is the median effect concentration for inhibition of growth based on a comparison of areas under the growth curves after "x" hours. The E_bC_{50} was calculated using a computer program by the moving average method (Stephan; 1977, 1982). The program uses percentage effect and the mean measured test concentrations in test samples.

The average specific growth rate (μ) for each exponentially growing culture is also calculated from the appropriate section of the growth curve by the equation:

$$\mu = \frac{\ln N_n - \ln N_o}{t_n - t_o}$$

Where t_o is the time at the beginning of the test.

The E_rC_{50} ("x" - "y" h) is the median effect concentration for inhibition of growth based on a comparison of growth rates from "x" to "y" hours. The E_rC_{50} was calculated by a computer program using either non-linear interpolation between the concentrations that bracket the 50% effect level or the moving average method (Stephan; 1977, 1982). The program uses the percentage effect and the mean measured test concentrations in test samples.

The "no observed effect concentration" (NOEC) was determined using Dunnett's multicomparison test to compare the percentage inhibition in the test group with that for the control cultures (Dunnett; 1955, 1964).

DEVIATION FROM PROTOCOL

For operational reasons, the medium in the preparation vessel used for the definitive test was left to stand for approximately 1.5 hours instead of 0.5 hours as stated in the study protocol.

This deviation to protocol is not thought to have affected either validity or integrity of the study.

MAINTENANCE OF RECORDS

All raw data arising from the performance of this study will remain the property of the Sponsor.

All raw data and study related documents generated during the course of the study at Huntingdon Life Sciences, together with a copy of the final report will be lodged in the Huntingdon Life Sciences Archive.

Records will be retained for a minimum period of ten years from the date on which the final report is issued. At the end of the ten year retention period the Sponsor will be contacted and advice sought on their future requirements. Under no circumstances will any item be discarded without the Sponsor's knowledge.

A retention sample as required under 40 CFR 792.105(d) was not taken nor held for the periods specified by 40 CFR 792.195 as the test substance was not indefinitely stable and posed a safety hazard.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

RESULTS

Chemical analysis

The results of chemical analysis are given in Table 1 and example chromatograms are illustrated in Figure 1.

Although lower than intended, the measured concentrations of 2-methyl-2-butene at the start (between 19 and 27% of their nominal values) were adequately maintained during the test, giving measured levels of between 22 and 29% of nominal after 96 hours. Based on an arithmetic mean, the overall mean measured levels of 2-methyl-2-butene were 0.689, 1.53, 3.61, 7.22 and 21.1 mg/l. The low levels measured during the study reflect the difficulties associated with testing a volatile substance.

After 96 hours, analysis samples of media containing 2-methyl-2-butene at 3.20 and 75 mg/l which had been incubated without algal cells gave slightly higher results than test media incubated in the presence of algal cells (30 and 33% of nominal without algal cells compared to 23 and 29% of nominal with cells; Table 1). These results suggest that the presence of algal cells may have had a small effect on the stability of the test substance under test conditions.

Algal growth

Individual cell densities for each culture and the mean values are given in Table 2. Three flasks on Day 2 and one flask on Day 3 showed anomalously low cell densities; the results from these flasks have been excluded from the test calculation. The reason for these low values was not identified.

The calculated area under the growth curve and average specific growth rate values are given in Table 3 and are expressed in terms of percentage inhibition by comparing the test group value with that of the control curve (Figure 2).

The test results have been expressed in terms of mean measured concentrations of 2-methyl-2-butene. The following values were derived from the data:

Area under the growth curve

E_bC_{50} (72 h) : 10.5 mg/l (95% confidence limits, 9.55 & 11.7 mg/l)

E_bC_{50} (96 h) : 10.1 mg/l (95% confidence limits, 9.21 & 11.1 mg/l)

No observed effect concentration (NOEC) : 3.61 mg/l

Average specific growth rate

E_rC_{50} (0 - 72h) : 12.0 mg/l (95% confidence limits, 7.22 & 21.1 mg/l)

E_rC_{50} (0 - 96 h) : 13.2 mg/l (95% confidence limits, 12.2 & 14.3 mg/l)

No observed effect concentration (NOEC) : 7.22 mg/l

Observations

After 96 hours of exposure, the majority of cells at 21.1 mg/l were swollen and/or mis-shapen.

Environmental parameters

The measurements of water quality (temperature and pH) in control and test flasks are summarised in Table 4. They remained within acceptable limits throughout the study although the pH of the control cultures increased by more than 1.5 pH units during the 96 hour exposure period; the test guidelines for this type of study recommend that the pH of control cultures should not deviate by more than 1.5 pH units during the test. The increase in pH observed during the definitive test was associated with the high level of cell growth that occurred but is not thought to have affected either the validity or integrity of the test because the validity criteria for this type of test were met by the control cultures.

The temperature of the incubator ranged between 22.3 to 23.4°C.

At the start of the test, the test media at all concentrations were clear and colourless with an odour detected at all levels.

CONCLUSIONS

After 72 and 96 hours of exposure to 2-methyl-2-butene, the E_bC_{50} values were 10.5 and 10.1 mg/l, respectively; the E_rC_{50} values were 12.0 and 13.2 mg/l respectively.

The "no observed effect concentration" (NOEC) for area under the growth curve and growth rate, respectively were 3.61 and 7.22 mg/l.

REFERENCES

DUNNETT, C.W. (1955) "A multiple comparison procedure for comparing several treatments with a control". Journal of the American Statistical Association, 50, 1096-1121.

DUNNETT, C.W. (1964) "New tables for multiple comparisons with a control". Biometrics, 20, 482-491.

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OECD Procedure 201 'Alga, Growth Inhibition Test' of the 'Guidelines for Testing of Chemicals', adopted 7 June 1984.

STEPHAN, C.E. (1977). "Methods for calculating an LC_{50}" in: MAYER, F.L. and HAMELINK, J.L. (Eds.) *Aquatic Toxicology and Hazard Evaluation*, p. 65-84. ASTM STP 634 American Society of Testing and Materials.

STEPHAN, C.E. *et al.* (1982). *Pers. Comm.* "A computer program for calculating an LC_{50}" submitted to ASTM Task Group on calculating LC_{50}s.

US EPA (TSCA), Title 40 Code of Federal Regulations, Part 797 – Environmental Effects Testing Guidelines, sub-part B – Aquatic Guidelines, §797.1050 "Algal acute toxicity test" and §797.1060 "Freshwater algae acute toxicity test", 1 July 1989.

FIGURE 1

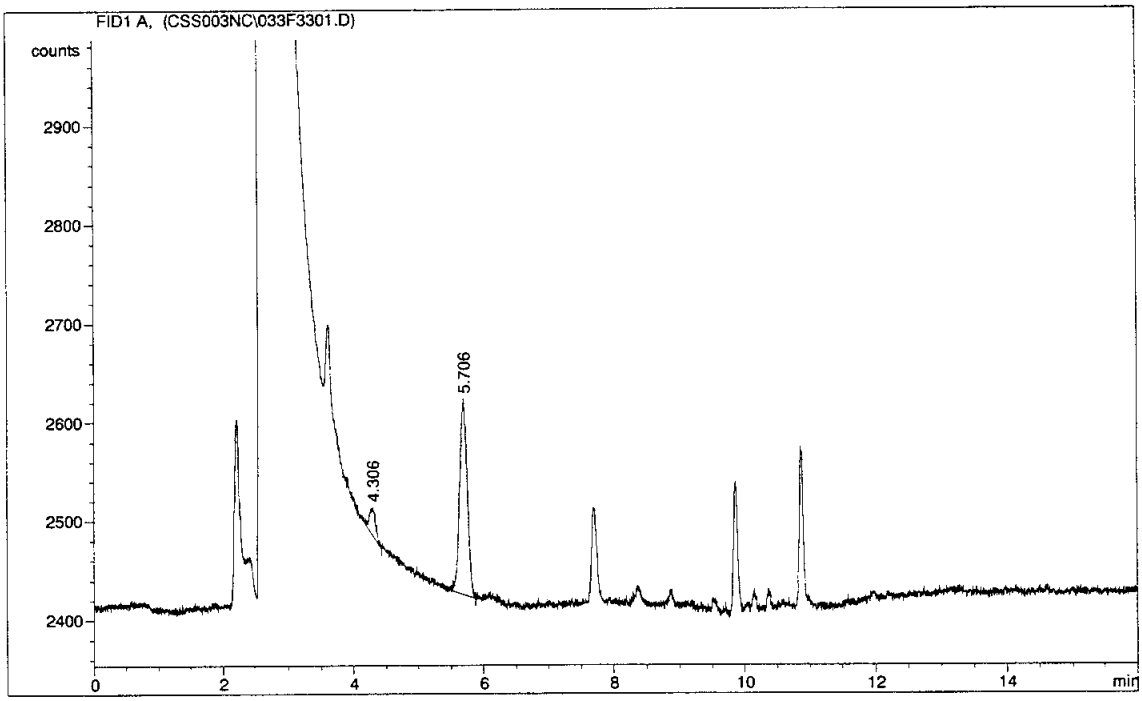
Sample chromatograms

a) 34.1 mg/l on Day 0

Data File C:\HPCHEM\2\DATA\CSS003NC\033F3301.D Sample Name: 34.1 A

Injection Date	: 16/11/02 06:43:46	Seq. Line	: 33
Sample Name	: 34.1 A	Vial	: 33
Acq. Operator	: nc	Inj	: 1
		Inj Volume	: 1 µl
Sequence File	: C:\HPCHEM\2\SEQUENCE\CSS003.S		
Method	: F:\CHEMST~1\METHODS\CSS003.M		
Last changed	: 12/11/02 10:02:41 by nc		

GC method for the analysis of 2M2B
Integration Events for table event_FID
Area reject 100.000, Height reject 5.000, Slope Sensitivity 50.000, Peak width 0.040, Shoulder off, 0.000 Stop Integration, 4.000 Start Integration, 6.500 Stop Integration



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [counts]	Height [counts]	Area %
1	4.306	VP	0.0727	176.51715	29.53183	9.88072
2	5.706	BP	0.1004	1609.96362	198.88081	90.11928
Totals :				1786.48077	228.41264	

*** End of Report ***

FIGURE 1 - continued

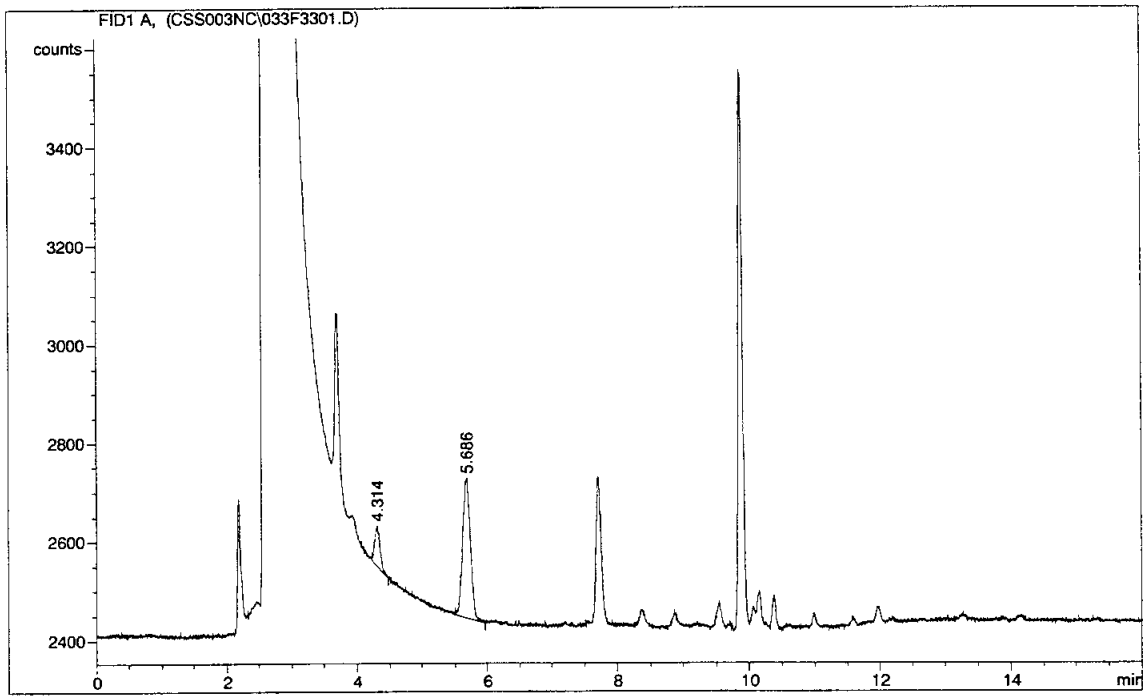
Sample chromatograms

b) 34.1 mg/l on Day 4

Data File C:\HPCHEM\2\DATA\CSS003NC\033F3301.D Sample Name: 4/34.1 A

Injection Date	: 19/11/02 06:37:19	Seq. Line	: 33
Sample Name	: 4/34.1 A	Vial	: 33
Acq. Operator	: nc	Inj	: 1
		Inj Volume	: 1 µl
Sequence File	: C:\HPCHEM\2\SEQUENCE\CSS003.S		
Method	: F:\CHEMST~1\METHODS\CSS003.M		
Last changed	: 15/11/02 17:03:09 by nc		

GC method for the analysis of 2M2B
Integration Events for table event_FID
Area reject 100.000, Height reject 5.000, Slope Sensitivity 50.000, Peak width 0.040, Shoulder off, 0.000 Stop Integration, 4.000 Start Integration, 6.500 Stop Integration



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [counts]	Height [counts]	Area %
1	4.314	BP	0.0719	469.37918	80.63116	16.62888
2	5.686	VP	0.0992	2353.29541	282.56296	83.37112
Totals :				2822.67459	363.19411	

*** End of Report ***

FIGURE 2

Inhibition of growth

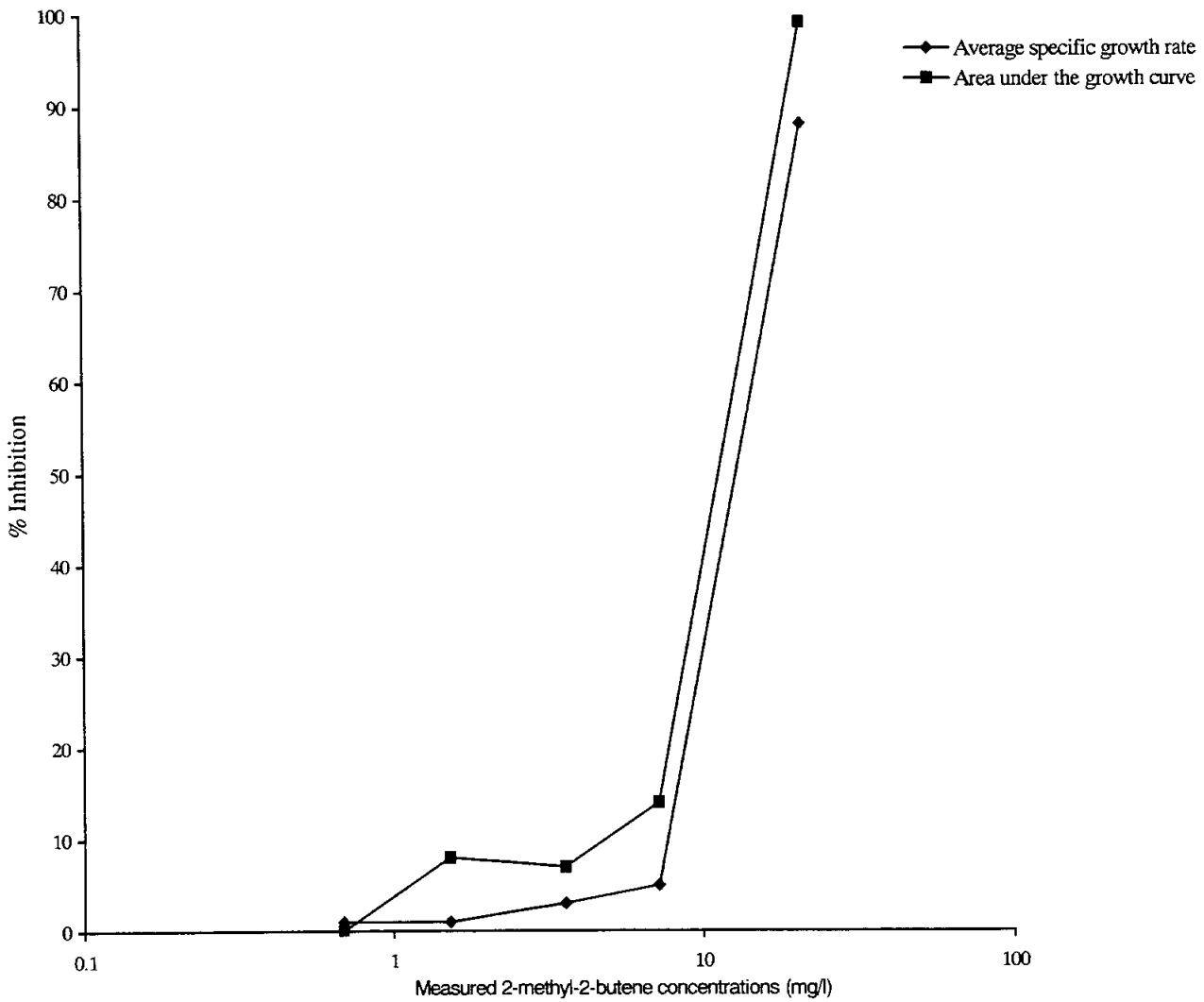


TABLE 1

Measured concentrations

Nominal conc (mg/l)	Measured 2-methyl-2-butene concentrations (mg/l) 0 hours		%N	96 hours		%N	% ti	Overall arithmetic mean
0	nd	nd	-	nd	nd	-	-	-
3.2	0.667	0.618	20	0.679	0.790	23	114	0.689 (22)
3.2A	-	-	-	1.03	0.877	30	148	-
7.04	1.42	1.59	21	1.64	1.45	22	103	1.53 (22)
15.5	3.73	3.98	25	3.52	3.21	22	87	3.61 (23)
34.1	5.90	7.26	19	8.02	7.70	23	119	7.22 (21)
75	20.2	20.3	27	19.9	23.9	29	108	21.1 (28)
75A	-	-	-	23.8	25.6	33	122	-
75_{WAF}	33.1	31.7	43	-	-	-	-	-

nd : not detected (< 0.7 mg/l).
% ti : mean measured concentration after 96 hours expressed as a percentage of the mean starting concentration.
%N : mean measured concentration (quoted to 3 significant figures) expressed as a percentage of the nominal concentration.
( ) : overall mean measured concentration (quoted to 3 significant figures) expressed as a percentage of the nominal concentration.
WAF : sample taken directly from the aqueous preparation before the vessel was opened and media removed to provide the test media.
A : test media without algae incubated under the test conditions.
- : no analysis performed / data not required.

TABLE 2

Cell densities

2-methyl-2-butene (mg/l)		Replicate	Cell densities (x 10^4cells/ml)			
Nominal	Mean measured	number	24 hours	48 hours	72 hours	96 hours
Control	nd	R_1	6.75	13.1	72.5	151
		R_2	2.75	15.0	13.5*	154
		R_3	5.63	32.9	68.6	145
		Mean	5.04	20.3	70.6	150
3.20	0.689	R_1	4.00	21.9	80.0	139
		R_2	2.75	3.63*	80.9	134
		R_3	5.38	27.3	67.5	152
		Mean	4.04	24.6	76.1	142
7.04	1.53	R_1	3.88	14.0	65.5	137
		R_2	3.50	15.6	62.8	147
		R_3	5.25	20.3	64.4	148
		Mean	4.21	16.6	64.2	144
15.5	3.61	R_1	3.25	2.75*	77.9	140
		R_2	5.88	11.8	79.0	128
		R_3	3.25	17.1	64.5	129
		Mean	4.13	14.5	73.8	132
34.1	7.22	R_1	3.25	17.1	69.1	114
		R_2	1.88	21.0	66.5	119
		R_3	2.75	3.75*	63.3	120
		Mean	2.63	19.1	66.3	118
75	21.1	R_1	1.63	1.50	1.13	2.38
		R_2	1.38	1.13	0.750	3.13
		R_3	1.00	1.25	1.00	0.875
		Mean	1.34	1.29	0.960	2.13

nd : not detected (< 0.7 mg/l).
R_1-R_3 : replicate number.
* : anomalously low values, omitted from calculation of the test results.

Note : mean cell density values are expressed to three significant figures.

TABLE 3

Inhibition of growth

2-methyl-2-butene (mg/l) Nominal	Mean measured	Replicate number	Area under curve at 72 h#	Area under curve at 96 h#	Growth rate (0-72 h)@	Growth rate (0-96 h)@
Control	nd	R_1	1286	3944	5.949	5.226
		R_2	1213	3884	5.913	5.247
		R_3	1688	4227	5.873	5.184
		mean	1396	4019	5.912	5.219
3.20	0.689	R_1	1522	4126	6.086	5.140
		R_2	1567	4122	6.102	5.102
		R_3	1534	4144	5.850	5.233
		mean	1541 (0)	4131 (0)	6.013 (0)	5.158 (1)
7.04	1.53	R_1	1155	3561	5.808	5.125
		R_2	1152	3646	5.750	5.198
		R_3	1326	3851	5.785	5.205
		mean	1211 (13)	3686 (8)	5.781 (2)	5.176 (1)
15.5	3.61	R_1	1301	3892	6.049	5.148
		R_2	1312	3772	6.069	5.054
		R_3	1202	3500	5.787	5.062
		mean	1272 (9)	3721 (7)	5.968 (0)	5.088 (3)
34.1	7.22	R_1	1258	3431	5.883	4.934
		R_2	1287	3489	5.829	4.978
		R_3	1224	3400	5.761	4.987
		mean	1256 (10)	3440 (14)	5.824 (1)	4.966 (5)
75	21.1	R_1	28.68	46.80	0.170	0.903
		R_2	9.240	31.80	-0.400	1.189
		R_3	6.000	4.500	0	-0.139
		mean	14.64 (99)	27.70 (99)	-0.077 (100)	0.651 (88)

\# : $x10^4$
@ : $x10^{-2}$
nd : not detected (< 0.7 mg/l).
R_1-R_3 : replicate number.
( ) : mean values expressed as % of the control cultures.

TABLE 4

Environmental parameters

a) Temperature and pH

2-methyl-2-butene (mg/l)		Temperature °C		pH	
Nominal	Mean measured	0 h	96 h	0 h	96 h
Control	nd	22.9	22.6	7.19	10.7
3.20	0.689	22.9	22.8	7.20	10.3
7.04	1.53	22.8	22.8	7.21	10.7
15.5	3.61	22.9	22.9	7.18	10.6
34.1	7.22	22.8	22.8	7.23	10.5
75	21.1	22.8	22.9	7.36	7.35

nd : not detected (<0.7 mg/l).

b) Light intensity

Test day	Lux measurements
0	4030
1	3430 → 4050*
2	3890
3	4360
4	4360

* : after measuring the light intensity of the test area on Day 1, it was increased to *ca.* 4000 lux.

APPENDIX 1

ALGAL NUTRIENT MEDIUM (OECD)

Four stock solutions were prepared according to the following table, using filtered, dechlorinated tap water which had been softened and treated by reverse osmosis, before microfiltration and purification (resistivity of 18 Megohm/cm). Stock solutions were sterilised by autoclaving (solutions 1-3) or by membrane filtration (solution 4) before being stored at 4°C in the dark.

Aliquots of stock solutions 1-3 were further diluted with the same diluent and autoclaved again to produce the working strength nutrient medium. Stock solution 4 was added to the medium on the day of use. The pH of the medium was adjusted to approximately 7 before use.

Nutrient	Concentration in stock solution (g/l)	Volume of stock solution per litre of final medium (ml)	Final concentration in test solution (mg/l)
Stock solution 1: macro-nutrients			
NH_4Cl	1.5	10	15
$MgCl_2.6H_2O$	1.2		12
$CaCl_2.2H_2O$	1.8		18
$MgSO_4.7H_2O$	1.5		15
KH_2PO_4	0.16		1.6
Stock solution 2: Fe-EDTA			
$FeCl_3.6H_2O$	0.08	1	0.08
$Na_2EDTA.2H_2O$	0.1		0.1
Stock solution 3: trace elements			
H_3BO_3	0.185	1	0.185
$MnCl_2.4H_2O$	0.415		0.415
$ZnCl_2$	3×10^{-3}		3×10^{-3}
$CoCl_2.6H_2O$	1.5×10^{-3}		1.5×10^{-3}
$CuCl_2.2H_2O$	10^{-5}		10^{-5}
$Na_2MoO_4.2H_2O$	7×10^{-3}		7×10^{-3}
Stock solution 4: $NaHCO_3$			
$NaHCO_3$	50	6	300

APPENDIX 2

THE DETERMINATION OF 2-METHYL-2-BUTENE IN AQUEOUS MEDIUM

SAMPLE ANALYSIS

The aqueous samples were diluted with methanol to bring the expected analyte concentration within the calibration range, at the same time adjusting the solvent composition to methanol : water (8 : 2 v/v). They were then analysed for 2-methyl-2-butene by gas liquid chromatography (GLC) using flame ionisation detection[1].

CHROMATOGRAPHY INSTRUMENTATION AND CONDITIONS

A gas liquid chromatography system comprising auto-sampler, oven, flame ionisation detector and data processing system was used.

Column	
Material	: Fused silica capillary
Type	: CP-SIL-5CB
Dimensions (l × id)	: 30 m x 0.53 mm
Film thickness	: 5 µm
Carrier gas	: Nitrogen
Flow	: 4.3 ml/min
Oven temperature settings	
Initial	: 40°C held for 6 mins
Rate	: 20°C/min
Final	: 140°C held for 5 mins
Injector	
Type	: Splitless
Injector purge	: On after 0.15 mins
Injection volume	: 1 µl
Temperature	: 115°C
Septum purge	
Gas type	: Nitrogen
Flow	: 3.1 ml/min
Detector	
Type	: Flame ionisation
Temperature	: 250°C
Makeup gas	: Nitrogen
Makeup flow	: 30 ml/min
Hydrogen	: 30 ml/min
Air	: 400 ml/min

Under the above conditions 2-methyl-2-butene chromatographed as a single peak with a retention time of approximately 5.7 minutes.

---

[1] The method of analysis was developed at Huntingdon Life Sciences Ltd., using information supplied by the Sponsor.

CALIBRATION SOLUTIONS

Calibration solutions were prepared with the batch (A0153320) of 2-methyl-2-butene that was used in the biological phase of the tests. Results are consequently expressed in terms of the test substance as supplied.

Working calibration solutions in the nominal range 0.1 to 21 mg/l were prepared by volumetric dilution with methanol : water (8 : 2 v/v) of a primary standard prepared in methanol.

CALCULATIONS

2-Methyl-2-butene concentrations were determined using external standards.

The peak area responses for 2-methyl-2-butene in the calibration standard chromatograms were power regressed against calibration standard concentrations. The 2-methyl-2-butene concentration in each sample was then calculated using the following equation:

$$\text{Measured concentration of analyte (mg/l)} = \sqrt[B]{\frac{y}{A}} \times F$$

Where:

	y	=	Detector response to analyte in test chromatogram.
	A and B	=	Constants derived from the power regression.
	F	=	Factor to take into account sample processing.

VALIDATION OF THE ANALYTICAL PROCEDURE

The analytical procedure was validated by determining the linearity of response of the analytical system, the specificity of the chromatographic analysis, the limits of detection and quantification, and the method's accuracy and precision.

During the course of the study the performance of the method was monitored by the analysis of quality control samples.

TABLE 1

Validation recoveries of 2-methyl-2-butene from fortified samples of aqueous medium

Medium	Fortification level (mg/l)	Recovery as a % of fortification level
OECD algal medium	84.4	108
	84.4	103
	11.7	99.4
	11.7	115
	0.492	115
	0.492	116
	Control	ND
	Control	ND
Overall mean (RSD)		109 (6.45)

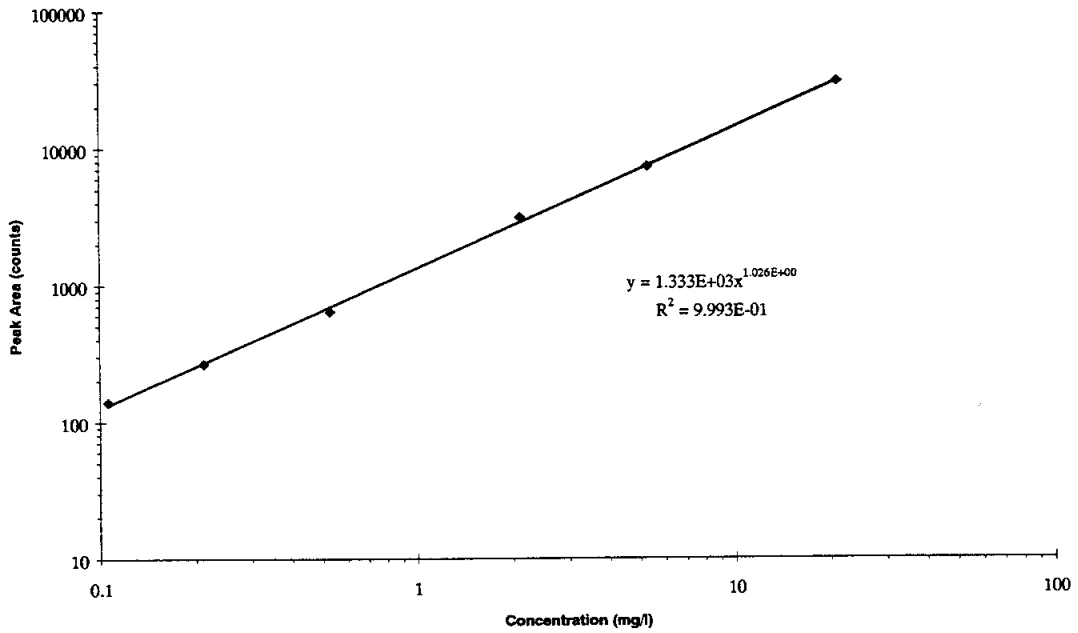
RSD: relative standard deviation.
ND: none detected; less than the limit of detection (0.7 mg/l).

The limit of detection is defined as the analyte concentration in a processed sample that would give a peak equal to 3 × local base-line noise.

The limit of quantitation is defined as the lowest successfully validated level (recoveries of 80 - 120 % with an RSD ≤ 20 %).

FIGURE 1

Standard calibration curve for 2-methyl-2-butene



Standard Conc. (mg/l)	Peak Area (Counts)	% Fit
21.2	30242	99.0
5.30	7299.6	99.0
2.12	3123.3	108
0.530	639.54	92.2
0.212	266.42	98.2
0.106	139.00	104

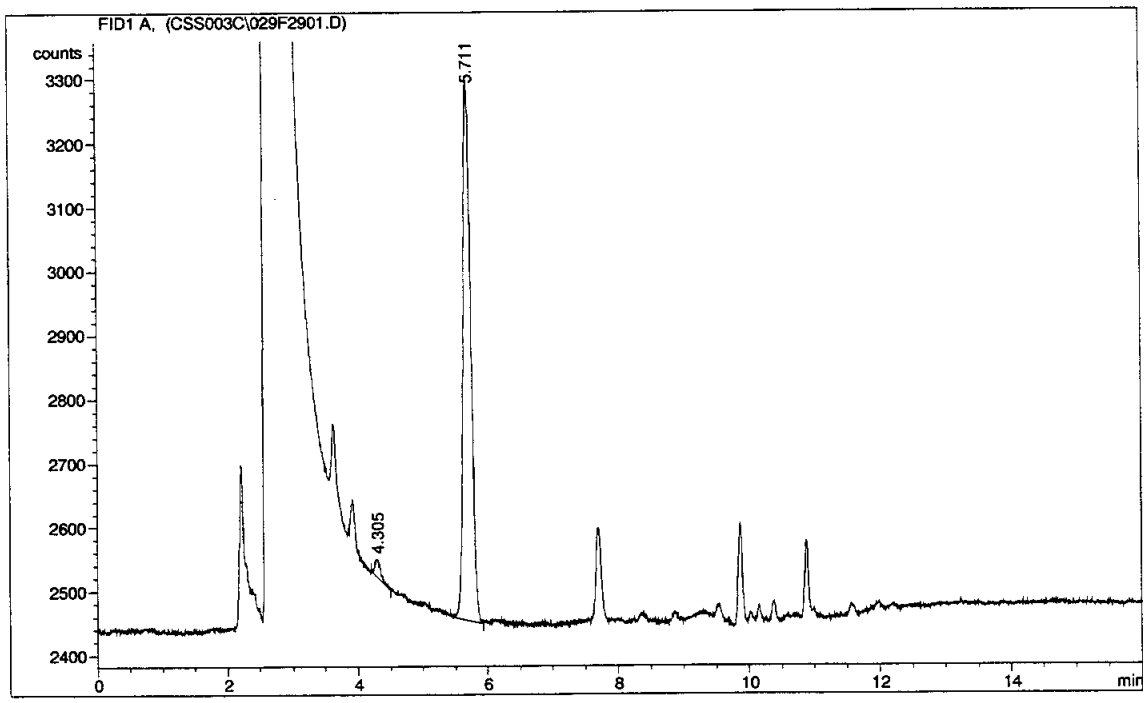
FIGURE 2

Representative chromatogram – 5.30 mg/l calibration standard

Data File C:\HPCHEM\2\DATA\CSS003C\029F2901.D Sample Name: STD (5.303 mg/l)

Injection Date	: 07/11/02 00:34:37	Seq. Line	: 29
Sample Name	: STD (5.303 mg/l)	Vial	: 29
Acq. Operator	: nc	Inj	: 1
		Inj Volume	: 1 µl
Sequence File	: C:\HPCHEM\2\SEQUENCE\CSS003.S		
Method	: F:\CHEMST~1\METHODS\CSS003.M		
Last changed	: 28/10/02 13:48:01 by nc		

GC method for the analysis of 2M2B
Integration Events for table event_FID
Area reject 100.000, Height reject 5.000, Slope Sensitivity 50.000, Peak width 0.040, Shoulder off, 0.000 Stop Integration, 4.000 Start Integration, 6.500 Stop Integration



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [counts]	Height [counts]	Area %
1	4.305	BP	0.0766	186.28099	29.12508	2.63930
2	5.711	VP	0.1136	6871.70117	838.85248	97.36070
Totals :				7057.98216	867.97756	

*** End of Report ***

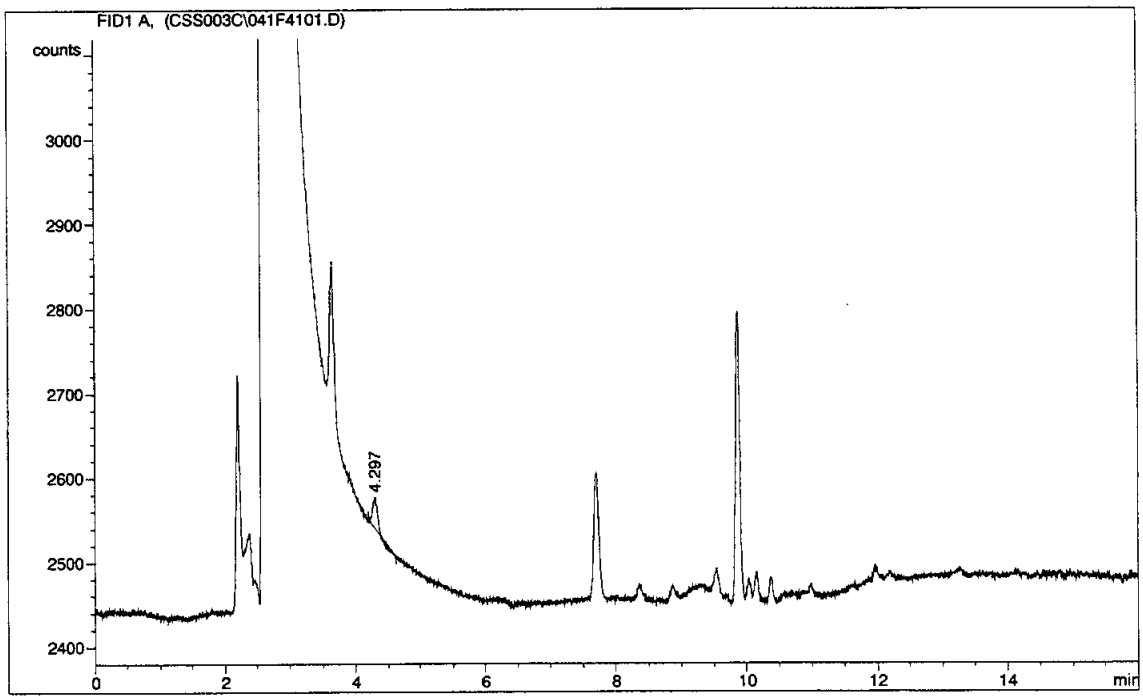
FIGURE 3

Representative chromatogram – OECD algal medium control

Data File C:\HPCHEM\2\DATA\CSS003C\041F4101.D Sample Name: CSP 0.00 A

Injection Date	: 07/11/02 05:37:17	Seq. Line	: 41
Sample Name	: CSP 0.00 A	Vial	: 41
Acq. Operator	: nc	Inj	: 1
		Inj Volume	: 1 µl
Sequence File	: C:\HPCHEM\2\SEQUENCE\CSS003.S		
Method	: F:\CHEMST~1\METHODS\CSS003.M		
Last changed	: 28/10/02 13:48:01 by nc		

GC method for the analysis of 2M2B
Integration Events for table event_FID
Area reject 100.000, Height reject 5.000, Slope Sensitivity 50.000, Peak width 0.040, Shoulder off, 0.000 Stop Integration, 4.000 Start Integration, 6.500 Stop Integration



Area Percent Report

Sorted By	:	Signal
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [counts]	Height [counts]	Area %
1	4.297	PP	0.0550	162.65588	35.82527	1.000e2
Totals :				162.65588	35.82527	

*** End of Report ***

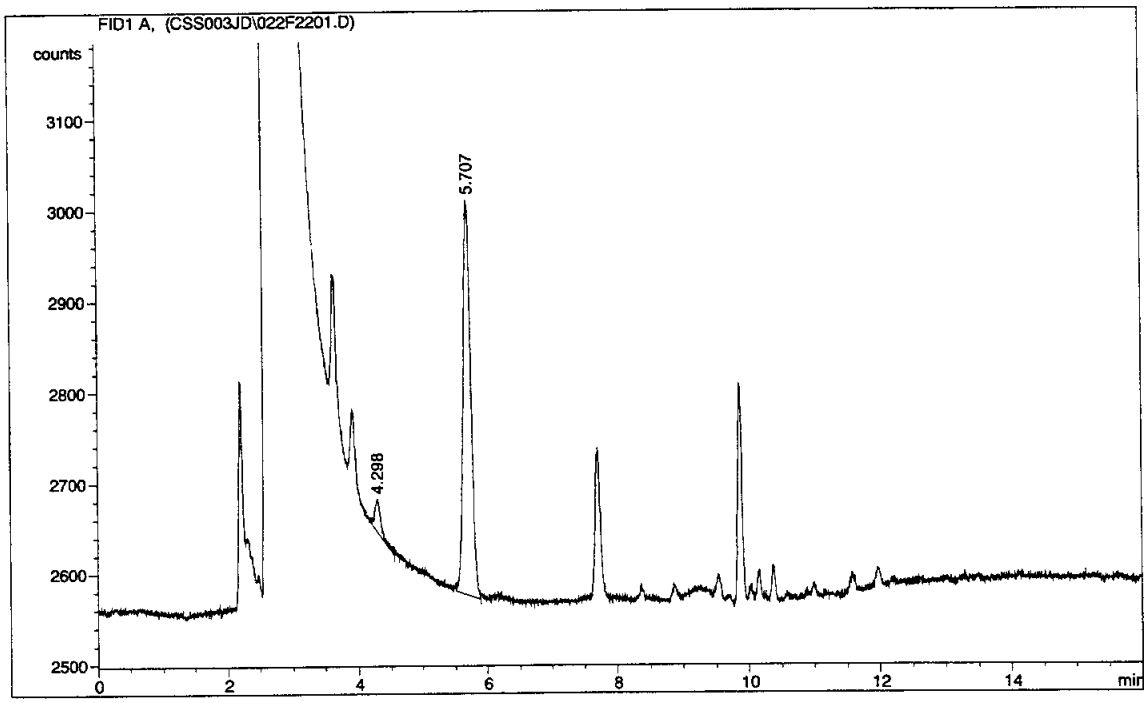
FIGURE 4

Representative chromatogram – sample of OECD algal medium fortified at 11.7 mg/l

Data File C:\HPCHEM\2\DATA\CSS003JD\022F2201.D Sample Name: CSP 16.4 A

Injection Date	: 12/11/02 20:00:16	Seq. Line	: 22
Sample Name	: CSP 11.7 A	Vial	: 22
Acq. Operator	: jd	Inj	: 1
		Inj Volume	: 1 µl

Sequence File : C:\HPCHEM\2\SEQUENCE\CSS003.S
Method : F:\CHEMST~1\METHODS\CSS003.M
Last changed : 12/11/02 10:02:41 by nc
GC method for the analysis of 2M2B
Integration Events for table event_FID
Area reject 100.000, Height reject 5.000, Slope Sensitivity 50.000, Peak width 0.040, Shoulder off, 0.000 Stop Integration, 4.000 Start Integration, 6.500 Stop Integration



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [counts]	Height [counts]	Area %
1	4.298	BP	0.0820	248.64388	36.71366	6.54136
2	5.707	VV	0.1053	3552.46265	434.76486	93.45864
Totals :				3801.10652	471.47852	

*** End of Report ***

APPENDIX 3

CERTIFICATE OF ANALYSIS

DEPARTMENT OF PRODUCT CHEMISTRY (HUNTINGDON)

HUNTINGDON LIFE SCIENCES

Test substance:	2-Methyl-2-butene
Batch number:	A0153320
Analysis dates:	Initial analysis: 2 August 2001 Final analysis: 12 December 2002
Data obtained as part of study:	CSS/007

Purity determined by GC analysis.

Purity:	Initial analysis:	98.0 %w/w
	Final analysis:	97.8 %w/w

Test substance stable for the duration of work performed at Huntingdon Life Sciences.

.....

John Betteley,
Study Director,
Huntingdon Life Sciences Ltd.

11 June 2003
.....
Date

APPENDIX 4

STUDY PROTOCOL AND AMENDMENTS

Study Number: CSS/003
Enquiry Number: 21329E

Huntingdon Life Sciences

CONFIDENTIAL

PROTOCOL

2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

Sponsor

American Chemistry Council
1300 Wilson Blvd.
Arlington
VA 22209
USA

Research Laboratory

Huntingdon Life Sciences Limited
Woolley Road
Alconbury
Huntingdon
Cambridgeshire PE28 4HS
ENGLAND

Total number of pages: 13

Study Number: CSS/003
Enquiry Number: 21329E

Huntingdon Life Sciences

PROTOCOL APPROVAL

2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

D.C Lodge
Dawn C. Lodge
Management
Huntingdon Life Sciences Ltd

17 July 2001
Date

Elizabeth J. Moran, Ph.D., D.A.B.T.
Sponsor's Representative for
American Chemistry Council

Aug 21, 2001
Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Study Director approval of the protocol is given on the study details page of the protocol once such details have been established and agreed. The completed page will be issued prior to the start of the study.

Study Number: CSS/003
Enquiry Number: 21329E

Huntingdon Life Sciences

STUDY DETAILS PAGE

Study number:	CSS/003
Study title:	Algal growth inhibition assay
Test species:	*Pseudokirchneriella subcapitata*
Test substance:	
Identity:	2-Methyl-2-butene
Lot number:	A0153320
Expiry date:	To be determined in HLS study no. CSS/007
Appearance:	Clear, colourless liquid
Storage conditions:	Cool, dry, well ventilated area
Purity/Assay:	98.2% (supplier); 98.0% in study CSS/007 (GC assay)
Supplier:	Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicester, LE11 5RG, UK.
Water solubility:	Insoluble
Sponsor's Representative:	Elizabeth J. Moran, Ph.D., D.A.B.T.
Head of Department:	Dawn C Lodge
Study Director:	Carole A Jenkins, B.Sc.
Person acting in the temporary absence of Study Director:	Deborah A Blacoe, B.Sc., Ph.D.
Location of study:	Department of Ecotoxicology, Building 33, Eye Research Centre, Eye, Suffolk, IP23 7PX, England
Proposed study dates	
Experimental start:	Week beginning 17 September 2001
Experimental termination:	Week beginning 7 December 2001
Draft report:	February 2002

STUDY DIRECTOR APPROVAL OF PROTOCOL

C A Jenkins — 14 September 2001

Carole A Jenkins, B.Sc.
Study Director
Huntingdon Life Sciences Ltd

Date

Study Number: CSS/003
Enquiry Number: 21329E

Huntingdon Life Sciences

CONTENTS

Study Number: CSS/003
Enquiry Number: 21329E

Huntingdon Life Sciences

1. INTRODUCTION

This test procedure is designed to determine the effects of test substances on exponentially-growing cultures of the unicellular green algae *Pseudokirchneriella subcapitata*. Solutions of the test substance in mineral salts culture medium and inoculated with a suspension of algal cells are continuously shaken, in completely-filled and sealed vessels, for 96 hours under static (no renewal) test conditions.

The effect of the test substance on the growth of the algal cultures, compared with controls, will be determined and the concentration which inhibits growth by 50% (EC_{50} - the median effect concentration or EL_{50} – median effect loading rate) calculated.

The study will comprise a range-finding test, followed by a definitive study to determine the EC_{50} / EL_{50} of the test substance to *Pseudokirchneriella subcapitata* with 95% confidence limits.

The study will conform to the principles for performing toxicity tests with aquatic organisms detailed in Procedure 201 of the "Guidelines for Testing of Chemicals" of the Organisation for Economic Co-operation and Development: " Alga, Growth Inhibition Test", adopted 7 June 1984 and Part C3 (Algal Inhibition Test) of the EC Methods for the Determination of Ecotoxicity: O.J. L383A (1992) with the following exceptions, so that the requirements of Sections 797.1050 and 797.1060 of the US EPA Code of Federal Regulations, Part 797 – Environmental Effects Testing Guidelines under the Toxic Substances Control Act (1987), can be met:

1. The duration of the test will be 96 hours.
2. The temperature of the test area will be 24 ± 2°C.
3. The light intensity of the test area will be approximately 4000 Lux.

Because the standard test design has been modified to accommodate the testing of volatile substances, the study will deviate from the recommendations of the OECD, EU and US EPA TSCA test guidelines in the following ways:

~~1. The initial algal cell density will be 1 x 10^4 cells/ml.~~
1.~~2~~. The pH of the culture medium will be 7.0 ± 0.2 unit

For test substances that are poorly soluble in water or are complex mixtures, water accommodated fractions (WAFs) will be prepared. Because complex test substances typically contain numerous components, each with a different solubility, quantification of the exposure concentrations in aquatic tests can be problematic and give results that are difficult to interpret. In view of this, the test results (EL_{50}) for such substances will be expressed in terms of the loading rates i.e. the nominal weights of test substance added to the culture medium at each exposure level and, where it is possible to verify the exposure levels analytically and the results are considered to be meaningful, the test results will also be expressed in terms of the mean measured concentrations.

The preparation of the WAFs will be based on the procedures outlined in the ASTM Standard Practice for Aquatic Toxicity Testing of Lubricants: Sample Preparation and Results Interpretation, D 6081, 1997.

Study Number: CSS/003
Enquiry Number: 21329E

Huntingdon Life Sciences

2. TEST ORGANISM

Unicellular green algae such as *Pseudokirchneriella subcapitata* are used as model systems in tests to determine whether a chemical affects the primary productivity of plants in the freshwater environment. Since many unicellular green algae have short generation times, this relatively short test can by used to assess the effects of a substance over many generations.

Cultures of *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum;* CCAP No. 278/4) will be obtained from the Culture Collection of Algae and Protozoa (CCAP), Ambleside, Cumbria, England.

3 MAINTENANCE AND PREPARATION OF CULTURES

Cultures of *Pseudokirchneriella subcapitata* will be maintained on nutrient agar under continuous illumination, in a refrigerator or at ambient temperature until required.

Before the study begins, autoclaved batches of mineral salts medium will be inoculated with algal cells taken from the agar cultures using a sterile loop: these (primary) liquid cultures (50 to 150 ml) will be placed in an illuminated orbital incubator at 24 ± 2°C and will be shaken for at least 72 hours to ensure that they are in the exponential phase of growth.

Appropriate volumes of primary cultures will be used to prepare secondary liquid cultures which will be incubated for a further 72 hours in an illuminated orbital incubator at 24 ± 2°C.

The inoculum produced in this way is typically in early to mid exponential growth with a cell density in the order of 10^5 - 10^6 cells/ml. The cell density of the secondary cultures will be determined before use.

4. DILUTION AND CULTURE MEDIUM

Tests will be conducted in the synthetic mineral salts medium detailed in the OECD guidelines. Each litre of the test medium will contain:

Nutrient salts	**Concentration (mg/l)**
NH_4Cl	15.0
$MgCl_2.6H_2O$	12.0
$CaCl_2.2H_2O$	18.0
$MgSO_4.7H_2O$	15.0
KH_2PO_4	1.6

Huntingdon Life Sciences

Nutrient salts	Concentration (mg/l)
$FeCl_3.6H_2O$	0.08
$Na_2EDTA.2H_2O$*	0.1
H_3BO_3	0.185
$MnCl_2.4H_2O$	0.415
$ZnCl_2$	0.003
$CoCl_2.6H_2O$	0.0015
$CuCl_2.2H_2O$	0.00001
$Na_2MoO_4.2H_2O$	0.007
$NaHCO_3$	50.0

* where appropriate, this will be omitted from the medium used in the study.

The medium will be prepared from a number of concentrated solutions. The stock solutions will be prepared in purified water and sterilised by autoclaving or by membrane filtration (0.2 µm pore size); they will be stored in the dark. The dilution medium will be allowed to equilibrate in air and, if necessary, the pH will be adjusted to approximately 7.0 before use.

Because the test is being conducted in completely filled and sealed vessels (with no headspace for gaseous exchange), sodium bicarbonate (*ca.* 300 mg/l) will be added to the culture medium used in the tests.

5. PREPARATION OF THE TEST SUBSTANCE

The identity, stability and purity of the test substance, and the amount and nature of any other components present are the responsibility of the Sponsor.

The exact methodology employed for preparing the test media to be used in the definitive test will be based on preliminary investigations and the range-finding test, and will be the subject of an amendment to protocol.

Where possible, the test media will be prepared individually at each concentration; if this is not feasible, then dilutions of a stock preparation or test media prepared at a higher concentration will be made.

The required volume of the test substance will be injected below the surface of the dilution medium in a preparation vessel (eg. glass bottle or aspirator, fitted with a glass sampling tube; capacity 1- or 2-l).

The volume added will be determined by weighing the syringe before and after the test substance is dispensed.

For poorly soluble substances and/or complex mixtures of substances, stirring of the dilution medium will commence before the test substance is added and immediately after the addition of the test substance, the preparation vessel will be sealed leaving no headspace. The test media will be stirred at a rate that is capable of generating a vortex of between 0 and 5% of the static depth of the medium in the preparation vessel before it is sealed. The preparation vessel will be covered to exclude light whilst it is being stirred. On cessation of stirring, each mixture will be left to stand in the test area for at least one hour and then approximately 100 ml will be withdrawn, via the sampling tube, from the middle of the vessel and discarded; finally, the required volume of the aqueous phase (water accommodated fraction: WAF) will be removed from mid-point in the vessel and used to fill the test vessels in the toxicity study.

The duration of the mixing period will be determined before the start of the toxicity study. Where possible, it will be determined by chemical analysis and will be based on the length of time it takes for the WAF to reach equilibrium although typically 20 to 24 hours of stirring will be adequate. Samples will be taken at intervals after stirring commences for a maximum of 96 hours.

If the WAFs prepared for use in the rangefinding test contain undissolved material (i.e. a micro-emulsion) and algal growth is inhibited, the definitive test may be conducted using the water-soluble fraction (WSF). The WSF will be prepared as stated above except that the WAF will be filtered before use. The decision to use a WSF rather than a WAF will be discussed with the Sponsor's Representative before the start of the definitive test and will be documented in an amendment to protocol.

The appearance of the aqueous mixtures and test media will be recorded during the study.

If there is a marked change in the pH of the culture medium after the addition of the test substance (outside the range 7.0 ± 0.2), the pH of the test media (or WAFs) may be adjusted by the addition of hydrochloric acid and/or sodium hydroxide. In such instances the Sponsor's Representative will be notified before the study start.

Decisions relating to the method of preparation of the test media will be made and verified by the Study Director before the start of the definitive study. All such decisions will be recorded in the raw data and subsequently reported in full.

6. TEST PROCEDURE

The study will be carried out in completely-filled, sealed vessels under static conditions without renewal of the test medium and will comprise at least one range-finding test, to provide an estimate of the toxicity of the substance, followed by a definitive test to define the relationship between concentration and effect.

In the range-finding test, algae will be exposed to solutions or WAFs of the test substance up to a maximum concentration or loading rate of 50 mg/l. At least two flasks of medium (*ca.* 50 ml per vessel) will be inoculated for each control and test group. Cell-density will be estimated after 96 hours using a haemacytometer.

The range of concentrations/loading rates used in the definitive test (at least five) will be based on the results of the range-finding test and will be arranged in a geometric series, with a spacing factor not exceeding 2.2, with a control. Where possible, exposure levels will be selected to give one at which no inhibition occurs and one in which growth is inhibited by at least 50% and preferably 100%.

An aliquot of the secondary algal culture will be added to the test medium prepared at each concentration to provide an initial cell-density of 1×10^3 cells/ml. Each vessel (glass conical flask; capacity *ca.* 50 ml) will be completely filled with inoculated test medium leaving no headspace and sealed with a glass stopper. The vessels will be labelled with the Study Number, vessel number and nominal exposure concentration/loading rate.

The control flasks will be prepared using the same procedures as used for the test vessels except that no test substance will be added.

At least ten flasks will be prepared for each control and test group in the definitive test. One flask or the remaining dilution (depending on the method of preparation) will be taken from each group at the beginning of the test and the temperature and pH of its contents measured: it will then be discarded. The remaining vessels in each group will be randomly allocated to positions in an illuminated orbital incubator, and incubated at a temperature of 24 ± 2°C with shaking at a speed of 150 rpm at a light intensity of approximately 4000 lux.

Cell-density will be estimated, at approximately 24, 48, 72 and 96 hours, using a haemacytometer. On each occasion, three vessels in each group will be removed from the incubator and samples removed for cell counts; if not required for chemical analysis or water quality measurements, these flasks will then be discarded.

The pH and temperature of the medium at each concentration will be measured at the end of the tests after the removal of samples for cell counting.

Incubator temperature and light intensity will be measured daily during the definitive test.

The concentration of the test substance in the test cultures will be verified using a GC method of chemical analysis at the start and end of the definitive test; where the test substance is known to be volatile, samples will be taken during the range-finding test at 0, 48 and 96 hours. Samples will be taken in duplicate from each concentration. In addition, analysis will be undertaken on preparations of the test substance at appropriate concentrations in culture medium, in the absence of algal cells and incubated under test conditions. Where possible, samples will be analysed without storage; if storage is necessary, the conditions will be outlined in an amendment to protocol.

7. RESULTS

The test results will be expressed in terms of nominal and the mean measured concentrations /loading rates of the test substance determined during the test. The 72- and 96-hour cell-density in test groups will be expressed as a percentage of the control for area under the growth curve and average specific growth rate.

Where possible, the median effect concentration or loading rate (EC_{50} or EL_{50} for average specific growth rate and area under the growth curve respectively) together with 95% confidence limits will be calculated by an appropriate statistical method. The EC_{10} and EC_{90} or EL_{10} and EL_{90} will also be quoted.

The 'no-observed effect concentration or loading rate' (NOEC or NOELR) will be calculated using an appropriate statistical method (e.g. Dunnett's test) to compare the percentage inhibition in each treated group with that for control cultures.

8. VALIDITY CRITERIA

For the test to be valid, the cell concentration in control cultures should have increased by a factor of at least 16 within 72 hours.

Growth at one test concentration/loading should be similar to the control group and one concentration should show a greater than 50% decrease in growth compared to the control.

9. GOOD LABORATORY PRACTICE

The study will be conducted in compliance with the principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

US EPA/TSCA Title 40 Code of Federal Regulations, Part 792, Federal Register, 17 August 1989.

The Study Director will normally seek approval of the Sponsor's Representative before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor's Representative, and for reasons of protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor's Representative.

Study Number: CSS/003
Enquiry Number: 21329E

Huntingdon Life Sciences

10. QUALITY ASSURANCE

The following will be inspected or audited in relation to this study.

Protocol Audit	:	The protocol.
Study Based Inspection	:	A critical phase of this study will be inspected.
Process Based Inspections	:	Routine and repetitive procedures will be inspected on representative studies, not necessarily on this study.
Report Audit	:	The draft report and study data will be audited before issue of the draft report to the Sponsor's Representative.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections which will be reported to appropriate Company Management only.

11. MAINTENANCE OF RECORDS

All raw data arising from the performance of this study will remain the property of the Sponsor.

A retention sample as required under 40 CFR 792.105(d) will not be taken nor held for the periods specified by 40 CFR 792.195 as the test substance is not indefinitely stable and may also pose a safety hazard. The identity, other characteristics and stability under conditions at the test site shall be documented by appropriate analyses prior to study start and completion.

All raw data will be retained by Huntingdon Life Sciences in its archive for a period of ten years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

12. HEALTH & SAFETY

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1994, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply

with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

13. REPORTING

The test report will include but not necessarily be limited to the following information:

Study management:

- the name of the test, sponsor, testing laboratory, study director and dates of testing.

Test substance:

- identification of the test substance and its purity;
- physical nature and, where relevant, physicochemical properties.

Test organisms:

- scientific name, strain number, origin, method of cultivation.

Test conditions:

- composition of the culture/dilution medium;
- apparatus and test conditions employed (e.g. methods of incubation, oscillation and illumination);
- methods of preparation of the test media and the concentrations used;
- water quality rates measurements undertaken during the study;
- description of test vessels, volume of medium in each vessel, initial cell density per vessel, number of test vessels per concentration/loading, the introduction of the test substance in the test medium;
- method for measuring cell density;
- methods of chemical analysis employed and the measurement undertaken during the study.

Results:

- individual and mean cell density at each sampling point (24, 48, 72 and 96 hours);
- appearance, size and colour of algal cells;
- growth curves in terms of the area under the growth curve and average specific growth rate;

Huntingdon Life Sciences

- percentage inhibition relative to the control group and a graphical presentation of the concentration-effect relationship;
- 96-hour EC_{50} / EL_{50} values (with 95% confidence limits, if possible);
- description or reference to statistical procedures applied;
- NOEC / NOELR, EC_{10} / EL_{10} and EC_{90} / EL_{90};
- results and date of a test performed with reference substance;
- all measurements of temperature and pH made during the study (tabulated);
- light intensity and oscillation rates;
- measured concentrations/loadings of the test substance during the study (tabulated);
- any protocol deviations and incidents in the course of the test that might have influenced the results.

An advance copy of the report will be used for comment by the Sponsor's Representative before the final report is printed. The authorised final report will include the information and data required by Good Laboratory Practice standards and the relevant test guideline reporting requirements and a copy of the Study Protocol. Unless otherwise requested, 4 bound copies of the final report will be issued on U.S. standard sized paper (quarto).

Corrections or additions to a final report will be in the form of an amendment by the Study Director. The amendment will clearly identify that part of the final report that is being added to or corrected and the reasons for the correction or addition, and will be signed and dated by the person responsible.

In the absence of ongoing communications, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional charge.

Enquiry Number : 21329E
Study Number : CSS/003
Protocol Amendment Number : 1

Huntingdon Life Sciences

2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

Total number of pages: 4

Number of pages for internal distribution: 4

Study Director : Carole A. Jenkins

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 10 February 2002
(Study Director)

For the Sponsor

Approved by: _____ Date: Feb 20, 2002

Enquiry Number : **21329E**
Study Number : **CSS/003**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

Reasons for amendment :

- Revision of the study timings;
- The algal slopes used for the study were supplied as aqueous cultures and not on agar;
- Based on the results of the range finding test, the amount of sodium bicarbonate added to the dilution medium will be reduced and the initial density of algal cells will be increased for the definitive test;
- Outline how the test media will be prepared for the definitive test and the nominal concentrations to be employed and;
- Revise the number of samples to be taken for chemical analysis.

AMENDMENTS:

STUDY DETAILS, page 3

Proposed study dates

Experimental start:	Week beginning 17 September 2001
Experimental termination:	Week beginning ~~7 December 2001~~ 19 February 2002
Draft report:	~~February~~ March 2002

1. INTRODUCTION, page 5, paragraph 6.

Because the standard test design has been modified to accommodate the testing of volatile substances, the study will deviate from the recommendations of the OECD, EU and US EPA TSCA test guidelines in the following ways:

~~1. The initial algal cell density will be 1 x 10^2 cells/ml.~~
1.2. The pH of the culture medium will be 7.0 ± 0.2 unit.

Enquiry Number : **21329E**
Study Number : **CSS/003**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

3. MAINTENANCE AND PREPARATION OF CULTURES, page 6, paragraphs 1 and 2.

Cultures of *Pseudokirchneriella subcapitata* will be maintained as aqueous cultures ~~on nutrient agar~~ under continuous illumination, in a refrigerator or at ambient temperature until required.

Before the study begins, autoclaved batches of mineral salts medium will be inoculated with algal cells taken from the aqueous ~~agar~~ cultures using a sterile pipette ~~loop~~: these (primary) liquid cultures (50 to 150 ml) will be placed in an illuminated orbital incubator at 24 ± 2°C and will be shaken for at least 72 hours to ensure that they are in the exponential phase of growth.

4. DILUTION AND CULTURE MEDIUM, page 7, paragraph 2

Because the test is being conducted in completely filled and sealed vessels (with no headspace for gaseous exchange), additional sodium bicarbonate (*ca.* ~~300~~ 250 mg/l) will be added to the culture medium used in the definitive tests.

5. PREPARATION OF THE TEST SUBSTANCE.

Page 7, paragraphs 2, 3 and 4

~~The exact methodology employed for preparing the test media to be used in the definitive test will be based on preliminary investigations and the range finding test, and will be the subject of an amendment to protocol.~~

~~Where possible, the test media will be prepared individually at each concentration; if this is not feasible, then dilutions of a stock preparation or test media prepared at a higher concentration will be made.~~

~~The required volume of the test substance will be injected below the surface of the dilution medium in a preparation vessel (eg. glass bottle or aspirator, fitted with a glass sampling tube; capacity 1 or 2 l).~~

Page 8, paragraphs 1 & 2

~~The volume added will be determined by weighing the syringe before and after the test substance is dispensed.~~

~~For poorly soluble substances and/or complex mixtures of substances, stirring of the dilution medium will commence before the test substance is added and immediately after the addition of the test substance, the preparation vessel will be sealed leaving no headspace. The test media will be stirred at a rate that is capable of generating a vortex of between 0 and 5% of the static depth of the medium in the preparation vessel before it is sealed. The preparation vessel will be covered to exclude light whilst it is being stirred. On cessation of stirring, each mixture will be left to stand in the test area for at least one hour and then approximately 100 ml will be withdrawn, via the sampling tube, from the middle of the vessel and discarded; finally, the required volume of the aqueous phase (water accommodated fraction: WAF) will be removed from mid-point in the vessel and used to fill the test vessels in the toxicity study.~~

For definitive test, the test media will be prepared, either directly or by dilution, from an aqueous preparation with a nominal concentration of 75 mg/l. The preparation vessel (amber glass bottle; total capacity *ca.* 2.68 l) will be completely filled with dilution medium and sealed with a silicone septum. The preparation vessel will be placed onto a stirrer and its contents gently stirred at a rate capable of generating a vortex of between 0 and 5% of the static depth of the mixture in the bottle before it was sealed. The test substance (nominally 305 µl) will be injected through the teflon septum into the dilution medium. The preparation vessel will be covered with a black bag and left stirring in the dark for approximately 24 hours. On cessation of stirring, the preparation will be left for approximately 30 minutes and after taking samples for chemical analysis, aliquots will be removed, via a sampling tube, from the middle of the vessel and used either to fill the test vessels at the highest concentration or further diluted in volumetric flasks to provide the test media at the lower concentrations.

6. TEST PROCEDURE, page 9, paragraphs 2, 3, 5 and 9

The ~~range of~~ nominal concentrations~~/loading rates~~ used in the definitive test will be 3.20, 7.04, 15.5, 43.1 and 75 mg/l. ~~(at least five) will be based on the results of the range finding test and will be arranged in a geometric series, with a spacing factor not exceeding 2.2, with a control. Where possible, exposure levels will be selected to give one at which no inhibition occurs and one in which growth is inhibited by at least 50% and preferably 100%.~~

An aliquot of the secondary algal culture will be added to the test medium prepared at each concentration to provide an initial cell-density of 1 x 10^{24} cells/ml. Each vessel (glass conical flask; capacity *ca.* ~~50~~ 65 ml) will be completely filled with inoculated test medium leaving no headspace and sealed with a glass stopper. The vessels will be labelled with the Study Number, vessel number and nominal exposure concentration/loading rate.

At least ~~ten~~ twelve flasks will be prepared for each control and test group in the definitive test. ~~One flask or~~ tThe remaining dilution in the preparation vessel ~~(depending on the method of preparation)~~ will be taken from each group at the beginning of the test and the temperature and pH of its contents measured: it will then be discarded. The ~~remaining~~ vessels in each group will be randomly allocated to positions in an illuminated orbital incubator, and incubated at a temperature of 24 ± 2°C with shaking at a speed of 150 rpm at a light intensity of approximately 4000 lux.

The concentration of the test substance in the test cultures will be verified using a GC method of chemical analysis at the start and end of the definitive test; where the test substance is known to be volatile, samples will be taken during the range-finding test at 0, 48 and 96 hours. Four sSamples will be taken ~~in duplicate~~ from each concentration; two will be analysed immediately and the others will be stored in case further analysis is required. Samples will also be removed from the aqueous preparation before use. In addition, analysis will be undertaken on preparations of the test substance at appropriate concentrations in culture medium, in the absence of algal cells and incubated under test conditions. ~~Where possible, samples will be analysed without storage; if storage is necessary, the conditions will be outlined in an amendment to protocol.~~

Enquiry Number : **21329E**
Study Number : **CSS/003**
Protocol Amendment Number : **2**

Huntingdon Life Sciences

2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

Total number of pages: 2

Number of pages for internal distribution: 2

Study Director : Carole A. Jenkins

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 19 August 2002
(Study Director)

For the Sponsor

Approved by: _____ Date: Sept 6, 2002

Enquiry Number : **21329E**
Study Number : **CSS/003**
Protocol Amendment Number : **2**

Huntingdon Life Sciences

2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

Reasons for amendment :

ξ The study timings have been revised because the definitive test is to be repeated;
ξ The number of samples to be taken for chemical analysis has been revised because analysis will not be conducted until completion of the definitive test.

AMENDMENTS:

STUDY DETAILS, page 3

Proposed study dates

Experimental start:	Week beginning 17 September 2001
Experimental termination:	Week beginning ~~19 February~~ 2 September 2002
Draft report:	~~March~~ October 2002

6. TEST PROCEDURE, page 9, paragraph 9

The concentration of the test substance in the test cultures will be verified using a GC method of chemical analysis at the start and end of the definitive test; where the test substance is known to be volatile, samples will be taken during the range-finding test at 0, 48 and 96 hours. ~~Four~~ Ssamples will be taken in duplicate from each concentration~~; two will be analysed immediately and the others will be stored in case further analysis is required~~. Samples will also be removed from the aqueous preparation before use. In addition, analysis will be undertaken on preparations of the test substance at appropriate concentrations in culture medium, in the absence of algal cells and incubated under test conditions. The samples will be retained in sealed, completely-filled, glass vials and the vials will be stored at approximately 40C until required.

Enquiry Number : **21329E**
Study Number : **CSS/003**
Protocol Amendment Number : **3**

Huntingdon Life Sciences

2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

Total number of pages: 2

Number of pages for internal distribution: 2

Study Director : Carole A. Jenkins

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 22 October 2002
(Study Director)

For the Sponsor

Approved by: _____ Date: Nov 5, 2002

Enquiry Number : **21329E**
Study Number : **CSS/003**
Protocol Amendment Number : **3**

Huntingdon Life Sciences

2-METHYL-2-BUTENE

ALGAL GROWTH INHIBITION ASSAY

Reasons for amendment :

- The study timings have been revised because the definitive test is to be repeated;
- Correction of the nominal exposure concentrations to be employed in the definitive test.

AMENDMENTS:

STUDY DETAILS, page 3

Proposed study dates

Experimental start:	Week beginning 17 September 2001
Experimental termination:	Week beginning ~~2 September~~ 4 November 2002
Draft report:	~~October~~ December 2002

6. TEST PROCEDURE, page 9, paragraph 2

The nominal concentrations used in the definitive test will be 3.20, 7.04, 15.5, ~~43.1~~ 34.1 and 75 mg/l.

APPENDIX 5

EYE RESEARCH CENTRE GLP COMPLIANCE STATEMENTS

29th January 2001

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

29th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger J. Alexander
3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

APPENDIX 5 - continued

EYE RESEARCH CENTRE GLP COMPLIANCE STATEMENTS

22nd April 2003

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Occold** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Toxicology** **Phys/Chem Tests**

DATE OF INSPECTION
22nd April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
25/7/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

APPENDIX 6

ROBUST SUMMARY

Algal growth inhibition assay

Test Substance	
Remarks	2-methyl-2-butene (CAS No. 513-35-9) also known as 2-methyl, 2-butene. The test substance was stable for the duration of all the studies performed by the test house.
Method	
Method/guideline followed	OECD Guide-line 201, EC Directive 92/69 C3, US EPA TSCA 797.1050 & 797.1060
GLP	Yes
Year	2003
Endpoint	Growth rate
Species	*Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) (Algae)
Exposure period	96 hours
Limit test	No
Analytical monitoring	Yes
Study Design	The study was conducted in completely filled (no headspace) and sealed vessels because of the volatility of 2-methyl-2-butene. The test media were prepared, either directly or by dilution, from an aqueous preparation in which the test substance was stirred in a sealed vessel for approximately 23 hours in the dark. After being allowed to stand for at least one hour to obtain an equilibrium concentration of 2-methyl-2-butene, aliquots of medium were removed from the middle of the vessel and after dilution and inoculated with algal cells, was used to fill the test vessels. The cultures were incubated in an orbital incubator under continuous illumination at temperatures ranging from 22.3 to 23.4°C for 96 hours. Replicate algal cultures, with an initial cell density of 1 x 10^4/ml, were exposed to 2-methyl-2-butene at nominal concentrations of 3.20, 7.04, 15.5, 34.1 and 75 mg/l. The exposure levels were monitored by measuring the concentrations of isoprene in samples of the test media using a GLC method of analysis. Cell densities were measured daily to monitor growth, and the test results are expressed in terms of the area under the growth curve and growth rate.
Evaluation of data	The area under the growth curve and the average specific growth rate are taken to be an index of growth and are calculated mathematically.

	The E_bC_{50} ("x" h) is the median effect concentration for inhibition of growth based on a comparison of areas under the growth curves after "x" hours. The E_bC_{50} was calculated using the moving average method of a computer program (Stephan:1977, 1982) which uses percentage effect and the nominal and measured test concentration in test samples. The E_rC_{50} ("x"- "y" h) is the median effect concentration for inhibition of growth based on a comparison of growth rates from "x" to "y" hours. The E_rC_{50} was calculated by either the moving average method or by non-linear interpolation between the two concentrations which bracket the 50% effect level of a computer program (Stephan:1977, 1982); the program uses percentage effect and the nominal and measured test concentration in test samples. The "no observed effect concentrations" (NOEC) was determined using Dunnett's multicomparison test to compare the percentage inhibition in the test group with that for the control cultures (Dunnett:1955, 1964).
Results	The measured concentrations of 2-methyl-2-butene ranged between 19 and 27% of their nominal values at the start of the test and between 22 and 29% of nominal after 96 hours. Based on an arithmetric mean, the overall mean measured levels of 2-methyl-2-butene were 0.689, 1.53, 3.61, 7.22 and 21.1 mg/l. Area under the growth curve (measured concentrations): E_bC_{50} (72 h) : 10.5 mg/l (95% confidence limits, 9.55 & 11.7 mg/l) E_bC_{50} (96 h) : 10.1 mg/l (95% confidence limits, 9.21 & 11.1 mg/l) No observed effect concentration (NOEC) : 3.61 mg/l Average specific growth rate (measured concentrations): E_rC_{50} (0-72 h) : 12.0 mg/l (95% confidence limits, 7.22 & 21.1 mg/l) E_rC_{50} (0-96 h) : 13.2 mg/l (95% confidence limits, 12.2 & 14.3 mg/l) No observed effect concentration (NOEC) : 7.22 mg/l
Observations	After 96 hours of exposure, the majority of the cells at 21.1 mg/l were swollen and/or mis-shapen.
Conclusions	After 72 and 96 hours of exposure to 2-methyl-2-butene, the E_bC_{50} values were 10.5 and 13.2 mg/l respectively; the E_rC_{50} values were 12.0 and 13.2 mg/l respectively. The "no observed effect concentration" (NOEC) for area under the growth curve and growth rate respectively, were 3.61 and 7.22 mg/l.
Data Quality	Valid without restrictions
Reference:	Huntingdon Life Sciences Ltd. 2003. Algal growth inhibition assay. Project ID CSS 003 Huntingdon Life Sciences Ltd., Cambridgeshire, England
Other Last changed	21 January 2004 Robust summary prepared by contractor to Olefins Panel